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14. ABSTRACT While bony metastases of prostate cancer are often osteoblastic, excessive bone resorption also occurs in the sites of metastases, which contributes to skeletal complications (e.g., pain, fractures). This research evaluates whether prostate cancer cells express the extracellular calcium (Ca ²⁺) _o -sensing receptor (CaSR) and whether the CaSR in bony metastases of prostate cancer participates in a vicious cycle involving CaSR-mediated secretion of the bone-resorbing cytokine, parathyroid hormone-related protein (PTHrP). The secreted PTHrP would promote further bone resorption, thereby increasing Ca ²⁺ _o locally and stimulating further PTHrP release. The project entails four tasks--namely showing that: (1) prostate cancer cells express the CaSR, (2) the CaSR mediates high Ca ²⁺ _o -induced stimulation of PTHrP secretion, (3) the CaSR transactivates the epidermal growth factor (EGF) receptor, and (4) CaSR-stimulated PTHrP secretion from prostate cancer cells increases the severity of metastatic bone disease in vivo in mice. We have accomplished tasks 1, 2, and 3 and are still working on developing the stably transfected cell lines needed for the studies in task 4. These results support a role for the CaSR in a vicious cycle that increases the severity of bone resorption <i>in vivo</i> in humans.					
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INTRODUCTION:

Prostate cancer research has generally emphasized the osteoblastic nature of prostate cancer metastases to bone. However, a wealth of recent data documents the nearly universal presence of excessive bone resorption as well, which participates importantly in the associated bone pain and fractures. The goal of this research is to determine whether prostate cancer cells express the extracellular calcium (Ca^{2+}_o)-sensing receptor (CaSR) and whether the CaSR participates in a vicious cycle promoting excessive bone resorption. This vicious cycle involves CaSR-mediated secretion of the bone-resorbing cytokine, parathyroid hormone-related protein (PTHrP), by prostate cancer metastatic to bone. The secreted PTHrP would produce further bone resorption, which would elevate the local level of Ca^{2+}_o , thereby stimulating further PTHrP release by the prostate cancer cells, and so forth. The scope of the project encompasses four specific aims: (1) to show that prostate cancer cells express the CaSR; (2) to prove that the CaSR mediates high Ca^{2+}_o -evoked stimulation of PTHrP secretion *in vitro*; (3) to determine whether the CaSR initiates a paracrine pathway producing transactivation of the epidermal growth factor receptor (EGFR), which then produces EGFR-mediated stimulation of MAPK and, in turn, increased PTHrP production; and (4) to document that CaSR-mediated stimulation of PTHrP secretion from prostate cancer cells injected into the femora of nude mice contributes to the severity of metastatic bone disease by knocking out the receptor using a dominant negative CaSR construct.

BODY:

Task 1—To document that prostate cancer cell lines express the CaSR (months 1-18).

We have completed the studies in task 1, which are described in detail in a publication of this work submitted with the previous Annual Report (1). A PDF file of this publication is also appended to the report. The results of these studies are as follows: Reverse transcriptase-polymerase chain reaction (RT-PCR) with intron-spanning primers amplified a product of the expected size, 480 bp, for having been derived from authentic CaSR transcript(s). In addition, Northern analysis, carried out using a CaSR-derived riboprobe and poly(A⁺) RNA derived from both LnCaP and PC-3 cells, revealed a major transcript of 5.2 kb, which is of the same size as the major transcript in human parathyroid gland (2).

With regard to documenting the presence of CaSR protein, immunocytochemistry with a polyclonal, CaSR-specific antiserum revealed specific staining of both PC-3 and LnCaP cells. Furthermore, western blotting with the same antiserum identified specific immunoreactive bands of 160-170 kDa in PC-3 and LnCaP cells, comparable in size to bands identified in the positive

controls--bovine parathyroid gland and CaSR-transfected human embryonic kidney (HEK293) cells (1).

Thus we have demonstrated that LnCaP as well as PC-3 cells express both CaSR transcript and protein. Note that while we originally proposed studies determining whether prostate cancer specimens removed at the time of prostatectomy expressed CaSR transcript(s) and protein, the contract for our grant expressly forbids the use of human anatomical substances.

Task 2--To show that the CaSR mediates the stimulation of PTHrP secretion from prostate cancer cell lines by high Ca^{2+}_o (months 6-24).

To investigate whether the CaSR mediated the stimulatory effect of high Ca^{2+}_o on PTHrP secretion from PC-3 cells (1), we utilized polycationic agonists (i.e., neomycin and spermine) known to activate the cloned CaSR (3, 4). The potencies of these two polycations were equal to or more effective than high Ca^{2+}_o in stimulating PTHrP secretion from PC-3 cells. We next used a naturally occurring, dominant negative construct of the CaSR (R185Q) to assess the CaSR's role in mediating high Ca^{2+}_o -evoked PTHrP secretion. To achieve high efficiency expression of the CaSR in PC-3 cells, we utilized infection with an adenoviral construct expressing the mutated CaSR. Compared to vector-infected cells, cells infected with the dominant negative CaSR showed a substantial reduction in the stimulation of PTHrP secretion by 1.5 and 3.5 mM Ca^{2+}_o (1), levels of Ca^{2+}_o that could potentially be encountered by bony metastases of prostate cancer near sites of active bone resorption (5).

Task 3--To investigate whether the CaSR transactivates the EGFR in prostate cancer cells (months 6-24).

In addition to the studies accomplished in tasks 1 and 2, we have shown that the CaSR transactivates the EGFR (see PDF file of the published paper appended to this report), thereby completing Task 3. Since the MAP kinase, ERK1/2, is a major signal transduction pathway utilized by the EGFR, we initially documented a delayed phosphorylation of ERK1/2 by Western blotting. Maximal activation was observed at 30 min, and a strong signal persisted at 60 min on Western blots of phospho-ERK1/2. At 120 minutes, in contrast, the signal had nearly dissipated. The phosphorylation of ERK1/2 was dose-dependent with regard to the level of Ca^{2+}_o employed; the strongest signal was observed with 7.5 mM Ca^{2+}_o , while signals of intermediate intensity were observed at 1.5 and 3.0 mM Ca^{2+}_o .

In order to document that high Ca^{2+}_o -evoked activation of ERK was CaSR-mediated, we examined the effects of the known polycationic CaSR agonist, spermine, and of a selective CaSR activator, NPS R-467, on phospho-ERK1/2. Incubation of PC-3 cells with 100 μM spermine for 30 min increased the level of phospho-ERK1/2. Moreover, NPS R-467 produced a much greater increase in phospho-ERK1/2 than

did its less potent stereoisomer, NPS S-467. Since NPS R-467 is 10 to 100 fold more potent than NPS S-467 in activating the CaSR, our results indicate that high Ca^{2+} -induced ERK phosphorylation is mediated by the CaSR.

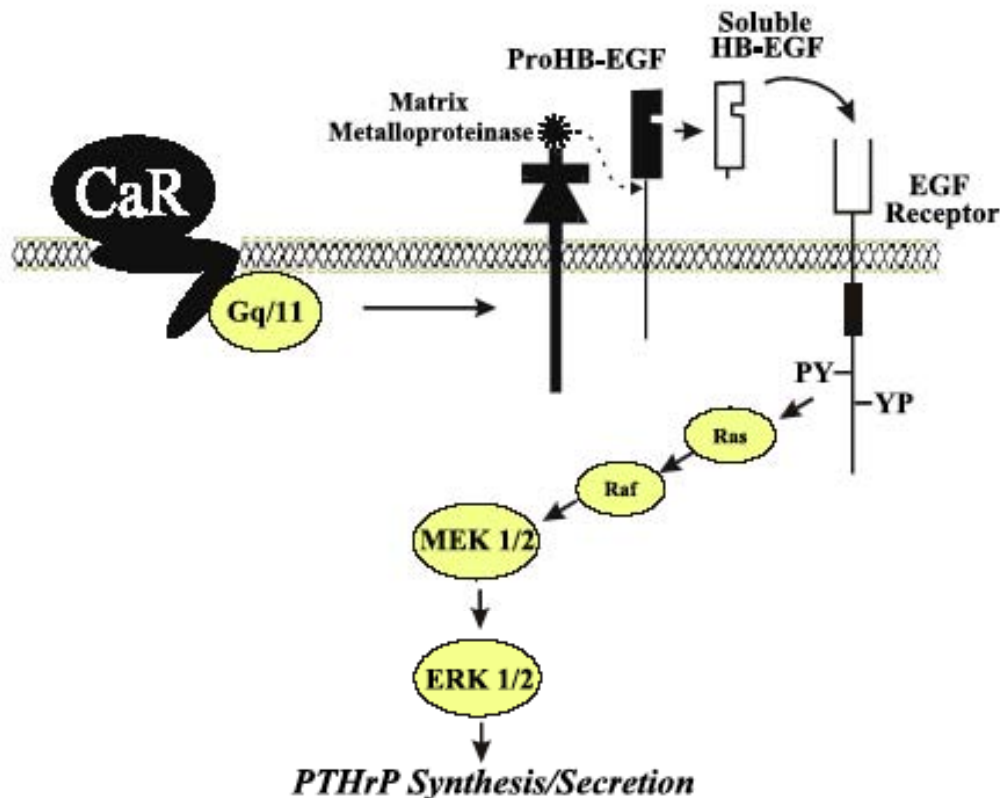
Next, we examined the effects of various inhibitors and neutralizing antibodies to assess the involvement of transactivation of the EGFR in CaSR-mediated activation of ERK1/2. AG1478, an EGFR kinase inhibitor, and PD98059, a MEK1 inhibitor, inhibited most of the high Ca^{2+} -evoked ERK phosphorylation. GM6001, a pan matrix metalloproteinase (MMP) inhibitor, and antibodies against the EGFR and HB-EGF (heparin-bound EGF) also reduced ERK phosphorylation, consistent with the model of transactivation shown on page 7 of this report. In contrast, AG1296, an inhibitor of the platelet-derived growth factor receptor kinase, had no effect on ERK phosphorylation. These results provide indirect evidence that activation of the CaSR transactivates the EGFR, but not the PDGFR, at least in part through activation of MMP(s).

We next directly measured the effect of high Ca^{2+} on the extent of phosphorylation of the EGFR. Phosphorylation of the EGFR was assessed using Western analysis with a monoclonal anti-phosphotyrosine antibody following immunoprecipitation of cell lysates with a rabbit polyclonal anti-EGFR antibody. The EGFR was phosphorylated to some extent even under basal (0.5 mM Ca^{2+}) conditions; following 10 min incubation in medium with 7.5 mM Ca^{2+} , however, the phosphorylation of the EGFR increased and was sustained for at least 30 min.

We have previously demonstrated that high Ca^{2+} stimulates PTHrP secretion from PC-3 cells (1). This action of Ca^{2+} is at least partially mediated by the CaSR, since hormonal secretion is reduced by transfecting the cells with a dominant negative CaSR, and known CaSR agonists, e.g., neomycin and gadolinium, promote PTHrP secretion (1). Thus, we wondered if the CaSR might stimulate PTHrP secretion through transactivation of the EGFR.

High Ca^{2+} dose-dependently stimulated PTHrP secretion by PC-3 cells. This stimulation was inhibited by 20 μM PD98059 and by 0.7 μM AG1478. In contrast, 1 μM AG1296 had no effect on PTHrP secretion. When the cells were preincubated with anti-HB-EGF antibody for 30 min, 5 $\mu\text{g/ml}$ of the antibody significantly inhibited PTHrP secretion (by 42%) even under basal conditions (0.5 mM Ca^{2+}). At 7.5 mM Ca^{2+} , the anti-HB-EGF antibody likewise produced a dose-dependent inhibition of PTHrP secretion. The anti-EGFR antibody gave similar results (data not shown). Preincubation with 10 μM GM6001 also reduced PTHrP secretion by 40% at 0.5 mM Ca^{2+} , and by about 50% at 3.0 and 7.5 mM Ca^{2+} . These findings indicate that EGF and HB-EGF activate the EGFR even under basal conditions and that high Ca^{2+} -induced PTHrP secretion is reduced by blockade of the CaSR-EGFR-ERK pathway. The former result is consistent with the presence of phosphorylated EGFR at 0.5 mM Ca^{2+} even following serum

starvation. These results are consistent with model shown below in which activation of the CaSR stimulates transactivation of the EGFR by activating a currently unidentified matrix metalloproteinase. The latter then cleaves heparin-bound EGF from its precursor, and the soluble HB-EGF activates the EGFR, thereby stimulating the activity of ERK1/2, likely by a ras and raf-dependent mechanism. The activated ERK1/2 then stimulates PTHrP secretion, which could participate in the feed-forward mechanism of enhanced bone resorption described above.



Task 4—To show that knocking out the CaSR reduces the severity of bone resorption in the femora of nude mice injected with PC-3 cells (months 6-36).

We continued during months 24-36 the development of PC-3 cells stably transfected with a dominant negative CaSR or with the corresponding vector. We have transfected PC-3 cells with a standard mammalian expression vector (pcDNA3) and subjected the transfected cells to selection with hygromycin. To date we have not yet been successful in obtaining individual, stably transfected PC-3 clones, in part because the cells grow very slowly at low density. While we have been able to select cells transfected with the dominant negative CaSR that grow in the presence of hygromycin, on immunocytochemistry only about 20% were positive for the CaSR. During the remaining few months of the grant, we will continue to develop individual clones of

stably transfected with the dominant negative CaSR so as to avoid the apparent heterogeneity in our studies to date. In addition to using the pcDNA3 vector, we will also try infecting the cells with the rAAV vector noted above and selecting for stably transfected cells.

KEY RESEARCH ACCOMPLISHMENTS:

- Documented the presence of CaSR transcripts in PC-3 and LnCaP cells as assessed by RT-PCR and Northern analysis.
- Demonstrated the presence of CaSR protein in PC-3 and LnCaP cells as assessed by immunocytochemistry and Western analysis.
- Shown that polycationic CaSR agonists stimulate PTHrP secretion from PC-3 cells, consistent with the CaSR's involvement in mediating high Ca^{2+}_o -evoked PTHrP secretion.
- Documented reduction of high Ca^{2+}_o -stimulated PTHrP secretion from PC-3 cells by infection of the cells with a dominant CaSR construct, supporting the CaSR's mediatory role.
- Shown that high Ca^{2+}_o and EGF stimulate ERK1/2 in PC-3 cells; Furthermore, the polycationic CaSR agonist, spermine, and the potent calcimimetic, NPS R-467, increase ERK1/2 in PC-3 cells to a greater extent than the less potent calcimimetic, NPS S-467, consistent with the mediatory role of the CaSR in this action.
- Demonstrated that an inhibitor of the EGF receptor kinase, a matrix metalloproteinase inhibitor, as well as antibodies against the EGFR and HB-EGF reduce high Ca^{2+}_o -evoked ERK activation, consistent with the involvement of CaSR-mediated transactivation of the EGFR, via matrix metalloproteinase-induced release of soluble EGF, in ERK1/2 activation.
- Shown that high Ca^{2+}_o -stimulated PTHrP secretion is reduced by the EGFR inhibitor, the matrix metalloproteinase inhibitor, and the antibodies to the EGFR and HB-EGF, providing further evidence that the CaSR transactivates the EGFR.
- Documented that high calcium stimulates a time dependent increase in the tyrosine phosphorylation of the EGFR, providing direct evidence for CaSR-mediated transactivation of the CaSR.

REPORTABLE OUTCOMES:

- a. Sanders JL, Chattopadhyay N, Kifor O, Yamaguchi T, Brown EM. Ca^{2+}_o -sensing receptor expression and PTHrP secretion on PC-3 human prostate cancer cells. Am J Physiol Endocrinol Metab 281:E1267-E1274, 2001.
- b. Yano S, Macleod RJ, Chattopadhyay N, Kifor O, Tfelt-Hansen J, Butters R, and Brown EM. Calcium Sensing Receptor Activation Stimulates Parathyroid Hormone Related Protein Secretion in Prostate Cancer Cells: Role of Epidermal Growth Factor Receptor Transactivation Bone 35:664-672, 2004.

CONCLUSIONS:

Our results support the major underlying hypotheses driving this research, namely that the CaSR mediates high Ca^{2+}_o -stimulated PTHrP secretion from PC-3 cells and could provide the basis for a "feed-forward" mechanism *in vivo* that would serve to aggravate the skeletal complications of prostate cancer metastatic to bone. The importance of this research lies in the implication that the CaSR could serve as a therapeutic target for CaSR antagonists that could diminish the severity of the skeletal complications of prostate cancer. Furthermore, it is possible that expression of the CaSR in other cancers that metastasize to bone (e.g., breast cancer) could serve as the mediator of a similar "feed-forward" mechanism and thereby provide the basis for a novel therapy of cancers other than prostate cancer.

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